SOLVENT PRODUCTION FROM PALM OIL MILL EFFLUENT (POME) BY CLOSTRIDIUM BEIJERINCKII

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ABSTRACT

Acetone, butanol and ethanol have widely been used in many industries. butanol and ethanol have been recognize as potential biofuel. While acetone is important as fuel tracer in field of combustion and use as suspension agent in pharmaceutical preparation. In 1990s, the increasing price of sugarcane have increased the production cost of ABE fermentation and the only factor that causing ABE fermentation could not survive was due to the cost of raw materials of substrate By using POME as substrate for fermentation process of acetone, butanol and ethanol (ABE), production is at advantage due to its lower cost and readily available. This study is to determine the effect of concentration of substrate, modified glucose concentration on substrate and initial pH of substrate towards enhancing solvent production from palm oil mill effluent (POME) by *Clostridium beijerinckii.* The experiment has been carried out in a batch culture system. The study of substrate pH effect ranged from 4.7, 5.2, 5.7 to 6.2. Meanwhile, for study of substrate concentration ranged from 60%, 70%, 80%, 90% to 100%. The study of modified substrate concentration effect were ranging from 20g/L, 30g/L, 40g/L, 50g/L to 60g/L. The fermentation of POME which produced acetone, butanol and ethanol, was analysed using gas chromatography flame ionization detector (GC-FID) and isobutanol was used as the internal standard. The experiment was using one factor at one time (OFAT) method to determine the best range values. From the parameters stated, sedimented POME at 70% concentration with initial pH increased to 5.7 is most suitable for solvent production 70% with 1.025g/L solvent produced. While 50g/L is the optimal concentration of modified glucose that enhanced the solvent production with supported by operating temperature at 37°c and 150rpm which 1.241g/L solvent produced. Apart from that, growth cell at pH5.7 maximize the solvent production where 0.967g/L solvent produced. In addition, DNS test for glucose consumption of all parameters studies can be conclude that higher glucose consumption resulted in higher amount of total solvent produced. The results obtained from these experiments showed that POME could be a viable media for ABE fermentation.

ABSTRAK

Aseton butanol dan etanol banyak digunakan didalam industry. Butanl dan etanol telah dikenalpasti sebagai nio-bahan api. Manakala asetn penting sebagai pengesan bahan api dalam bidang pembakaran dan digunakan dalam agen pemendapan dalam penyediaan farmaseutical. Sehubungan dengan ini, penapaian mikrob butanol adalah dianggap sebagai sumber bahan api cecair yang berpotensi. Dengan menggunakan POME sebagai substrat untuk proses penapaian aseton, butanol dan etanol (ABE), pengeluaran adalah lebih bermanfaat kerana kos yang jauh lebih rendah dan sentiasa tersedia. Kajian ini adalah untuk mengkaji kesan pH ke atas substrat (POME), kepekatan substrat (POME) dan kepekatan substrat yang diubah suai (POME) ke arah penghasilan pelarut dari bahan buangan minyak sawit (POME) oleh *C. beijerinckii*. Eksperimen telah dijalankan dalam sistem 'batch culture'. Kajian kesan pH substrat adalah terdiri daripada 4.7, 5.2, 5.7 , 6.2 sehingga 6.7. Sementara itu, untuk kajian kepekatan substrat adalah dari 60%, 70%, 80%, 90% kepada 100%. Kajian kesan kepekatan substrat diubah suai dari 20g/L, 30g/L, 40g/L, 50g /L 60g /L. Penapaian POME menghasilkan aseton, butanol dan etanol akan dianalisis menggunakan gas kromatografi dengan pengesan pengionan nyalaan (GC-FID) dan isobutanol telah digunakan sebagai piawai dalaman. Eksperimen menggunakan kaedah salah satu faktor pada satu-satu masa (OFAT) untuk menentukan nilai-nilai julat yang terbaik. Dari parameter yang dinyatakan, kepekatan POME adalah pada 70% dengan pH awal meningkat kepada 5.7 dengan penghasilan 1.025g/L pelarut. Sementara itu, 50g/L merupakan kepekatan optimum glukosa yang diubah suai untuk meningkatkan pengeluaran pelarut dengan suhu operasi pada 37 ° C dan 150rpm menghasilkan 1.241g/L pelarut. Selain daripada itu, pertumbuhan sel pada pH5.7 memaksimumkan pengeluaran pelarut di mana 0.967g/L pelarut yang dihasilkan. Di samping itu, eksperimen DNS untuk penggunaan glukosa untuk semua parameter boleh disimpulkan bahawa penggunaan glukosa yang lebih tinggi menghasilkan jumlah pelarut yang lebih tinggi dihasilkan. Keputusan yang diperolehi daripada eksperimen menunjukkan bahawa POME boleh menjadi media yang berdaya maju untuk penapaian ABE.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

CO_2	Carbon dioxide
N_2	Nitrogen
O_2	Oxygen
pH	Negative logarithm of the hydrogen ion concentration
C_4H_7OH	Molecular formula for butanol
C_2H_5OH	Molecular formula for ethanol
C_3H_6O	Molecular formula for acetone

LIST OF ABBREVIATIONS

ABE	acetone-butanol-ethanol
DNS	Dinitrosalicylic acid
FID	Flame Ionization Detector A
GC	Gas Chromatography
OD	Optical Density
OFAT	One Factor at One Time
POME	Palm Oil Mill Effluent
RCA	Reinforced Cultured Agar
RCM	Reinforced Cultured Medium
SEM	Scaning Electron Microscopy

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Palm oil also is widely used as cooking oil, soaps, washing powders, personal care products and biofuel. These productions generate waste which is palm oil mill effluent (POME). POME consisting of complex vegetative matter is thick, brownish, colloidal slurry of water, oil and solids including about 2% suspended soils (Bek Nielsen, *2007*). Due to its high biochemical oxygen demands about 25 000mg/l, chemical oxygen demands 53 630mg/l, oil and grease and total solid, POME was not allowed to be discharged into watercourse without any treatment (Wu *et al., 2009*). Apart from that, the effluent consist high composition and concentration of carbohydrate, protein, nitrogeneous compounds, lipid and mineral in POME are the specialty for the effluent to reuse through biotechnological advance. Furthermore, sedimented POME contains high concentration of lignocelluloses and other insoluble material that supported the growth of clostridia family (Takriff *et al, 2009*).

Utilization of POME as alternative sources of renewable energy and valuable chemical generate an advantage as POME contained many nutrients that suitable for production of biohydrogen, bioethanol, citric acid, and acetone-butanol-ethanol (ABE) fermentation. Apart of POME as substrate to produce ABE, others renewable resources including molasses, corn, whey permeate, wheat straw (WS), corn stover, corn fiber (Qureshi *et al., 2008)* are able to produce ABE. According to Mariano *et al.*, 2010, ABE fermentation are produced in the ratio 3:6:1 where the major production is butanol. Bio-ethanol blended with fuel would improve the performance of spark ignition in automotive engine (Park *et al.*, 2011). In addition, bio-ethanol blended gasoline can reduced the fuel cost of automotive with increasing ethanol content compared to regular unleaded gasoline (Yoo *et al.*, 2011). However, butanol has been reported to have better chemical properties to be superior fuel than ethanol (Qureshi *et al.*, 2008). In addition, acetone also plays an important role in the field of combustion as fuel tracer (Pichon *et al.*, 2009).

ABE fermentation using anaerobic bacterium *C.beijerinckii* has attract attention as chemical feedstock or liquid fuel replacement and the fermentation has been started during World War 1 which at that time, maize was used as the substrate (Gutierrez *et al.*, 1998). *Clostridium beijerinckii*, included in the genus Clostridium, is a commercially valuable bacterium and the strain has been used widely in producing bio-fuel production as a gasoline and diesel fuel replacement.

1.2 Problem Statement

A number of studies have suggested the cost of substrate is a major factor that influences the production cost of bio-fuels. In 1990s, the increasing price of sugarcane have increased the production cost of ABE fermentation and the only factor that causing ABE fermentation could not survive was due to the cost of raw materials of substrate as cost of substrate in fermentation will be about 60% of the overall cost (Kalil *et al.*, 2003)

In 1980s, the reduce supply and increasing of petroleum rekindled interest in fuel production by anaerobic bacteria including ABE fermentation. One large scale fermentation using *C acetobutylicum* strain was believe to be economically (Gheshlaghi *et al.*, 2009). However, the ABE fermentation could not be economically because of low yield of butanol due to its hetero-fermentative characteristic. Hetero-fermentative cause limitation of production due to its toxicity and cell inhibit growth that very sensitive to pH control and sugar concentration (Wang and Blashek., 2011).

1.3 Objective of Study

The objective of this study is to study the effect of concentration of substrate, modified glucose concentration on substrate and initial pH of substrate on solvent production by *Clostridium beijerinckii*.

1.4 Scope of Study

I. To study the growth profile of *Clostridium beijerinckii*.
II. To study the effect of concentration of substrate ranging from 60% to 100% on solvent production
III. To study the effect of modified glucose concentration on substrate ranging from 20g/L to 60g/L on solvent production
IV. To study the effect of initial pH of substrate ranging from 4.7 to 6.7 on solvent production
V. To study the glucose consumption at different concentration of substrate, different modified glucose concentration of substrate and different initial pH of substrate.

1.5 Significant of Proposed Research

The increasing of industrialization and motorization of the world has led to a steep rise for the demands of petroleum-based fuel. World urgently need to develop alternative fuel sources which can be produced from available resources. Bio-ethanol and bio-butanol are known as potential alternative fuel from bio-based resources and their characteristics provide the potential of reduction of particulate emission (Argawal *et al.*, 2007).

From economic viewed, fermentation of acetone, butanol and ethanol has been utilized by glucose, starch and corn. However, these substrate for ABE production are costly (Takriff *et al., 2009*). Thus by converting waste stream such as POME into higher value of ABE product from lower cost of source that contained many nutrient to support the growth of bacteria , would lead for profitability to the industries.

ABE fermentation with butanol as major product along with small amount of acetone and ethanol is produced biologically from renewable sources by *C. beijerinckii* under strictly anaerobic fermentation. ABE fermentation using clostridia continues to attract attention as those products produces the solvent of acetone, butanol and ethanol are commonly used solvent in many important industries (Tran *et al.*, 2010).

CHAPTER 2

LITERATURE REVIEW

2.1 ABE fermentation

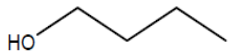
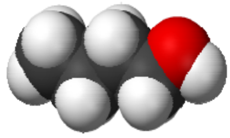
The anaerobe fermentation product produces the solvent acetone, butanol and ethanol with ratio 3:6:1 with the major product is butanol and the minor product is ethanol. ABE fermentation is a valuable product since all the product can be used in industries. Since 1980, efforts have been made to improve the ABE production by delineating the physiology of solventogenic clostridia, especially *C. acetobutylicum* and genetics (Gheshlaghi *et al*., 2009). New inventions have been developed on microbial fermentation by lower cost of substrate especially POME (Takriff *et al*., 2009), (Kalil *et al*., 2003) and corn waste (Qureshi *et al*., 2006). Microbial fermentation for ABE production has gained attraction of many researchers. Tran *et al*., 2010, produce ABE production from starch by mixed culture of *Bacillus subtilis* and *Clostridium butylicum*. The benefit of used mixed cultured enhance the ABE production. While Al-Shargoni *et al*., (2011) have investigated on various carbohydrate sources on ABE production using microbial fermentation of *C. saccharoperbutylacetonicum*. The result show that 50g/L of glucose medium produce highest production of ABE with higher amount of bio-butanol.

2.1.1 Butanol

C_4H_9OH is a molecular formula for butanol and the molecular weight is 74.12 g/mol. Butanol also known as butyl alcohol, n-butanol or methylolpropane. Butanol is a colourless, flammable slightly hydrophobic liquid with a distinct banana-like aroma and strong alcoholic odor. Hence, it is advised to use mask as the vapor may effect on mucous membranes and narcotic when inhale in high concentration. Butanol is less volatile than water. Because of that, distillation process to obtain butanol were not preferable for separating butanol from other component. For economic reason, fermentation process is more preferable because it provide efficient butanol recovery and economically. (Liu *et al*, 2004)

Characteristic of butanol determine itself as a potential for a better fuel extender or oxygenate than ethanol. Physically, butanol is more valuable than ethanol as it possesses many favourable properties including higher energy content, low vapour pressure, higher boiling point and miscibility with gasoline or fuel compared to ethanol (Tran *et al.*, 2010). Table 2.1 showed that the content of energy of butanol were 110 000 BTU/gal which is higher than only 84 000 Btu/gal for ethanol. This means butanol consumption is close to the pure gasoline. If compared to ethanol-gasoline blends with butanol gasoline-blends, ethanol-gasoline blends consumed much faster to obtain the same power input (Gholizadeh, 2009). It is mean, using less butanol is adequate. Because of this small consumption, the emission of carbon dioxide (CO_2), hydrocarbon will be reduced. Thus, it will result in positive impact for global environment.

Table 2.1 showed that the butanol has less volatility than ethanol advantage in less explosiveness. Butanol is much safer because of its high flash point and lower vapour pressure. Vapour pressure of butanol is 4 mmHg while 45 mmHg for ethanol. 11 times lower than ethanol enabling butanol to directly added to gasoline. Butanol is complete miscible gasoline that allows butanol to dispersed through existing pipeline. (Liu *et al,*. 2010). It also lower hygroscopicity which is not readily absorb moisture. Hence, butanol less affected by weather changes (Gholizadeh, 2009). Fermentation to obtain butanol is more preferable than distillation. It is because fermentation does not necessitate supplementation of all nutrient required by the culture (Ezeji *et al.*, 2007).

Properties	Butanol			Chemical Structure
Melting point (°C)	- 89.3			 1-butanol 
Specific gravity	0.810– 0.812			
Ignition temperature (°C)	35–37			
Auto-ignition temperature (°C)	343–345			
Flash point (°C)	25–29			
Relative density (water: 1.0)	0.81			
Critical pressure (hPa)	48.4			
Critical temperature (°C)	287			
Explosive limits (vol. % in air)	1.4–11.3			
Water solubility	9.0 ml/100 ml (7.7 g/100 ml at 20°C)			
Relative vapor density (air: 1.0)	2.6			
Vapor pressure (kPa at 20°C)	0.58			
	Butanol	**Gasoline**	**Ethanol**	**Methanol**
Boiling point (°C)	117–118	27–221	78	64.7
Density at 20°C (g/ml)	0.8098	0.7–0.8	0.7851	0.7866
Solubility in 100 g of water	immiscible	immiscible	miscible	miscible
Energy density ($MJ \cdot l^{-1}$)	27–29.2	32	19.6	16
Energy content/value (BTU/gal)	110000	115000	84000	76000
Air-fuel ratio	11.2	14.6	9	6.5
Heat of vaporization (MJ/kg)	0.43	0.36	0.92	1.2
Liquid Heat capacity (Cp) at STP (kJ/k-mol.°K)	178	160–300	112.3	81.14
Research octane number	96	91–99	129	136
Motor octane number	78	81–89	102	104
Octanol/Water Partition Coefficient (as $\log P_{o/w}$)[a]	0.88	3.52±0.62	-0.31	-0.77
Dipole moment (polarity)	1.66	n.a.	1.7	1.6
Viscosity (10^{-3} Pa.s)	2.593	0.24–0.32	1.078	0.5445

[a] LogP is a measure of hydrophobicity (lipophilicity) and is similar to polarity. These published values were obtained from Hansch *et al.*, (1995) for the three alcohols. In gasoline the LogP was roughly estimated as the weigh average of main representative components.

Table 2.1: Physical and Chemical Properties of butanol (adapted from Gholizadeh, 2009)

2.1.2 Ethanol

Ethanol, C_2H_5OH also known as ethyl elcohol, pure alcohol, grain alcohol or drinking alcohol. Beside that alcohol is colourless, volatile, flammable and has slight odor Ethanol has widely used in industries to produce fragrance, flavouring, colouring, medicines and in mid-1970s as a fuel for internal combustion engine.

In 2006, US has lead as larger producer of ethanol as ethanol production in US expanding rapidly. In US, ethanol was produced from corn and reported that 97% total ethanol production in US was produced from corn as substrate (Hettinga *et al.*, 2009). Lv Xing-Cai *et al.*, (2003) state that ethanol is a renewable energy that can be made from all kinds of raw materials such as sugar cane, molasses, cassava, sorghum, corn. Barley and waste biomass materials by using already improved and demonstrated technology. Ethanol is widely used as fuel for engine in Brazil, France, and Turkey. The advantage of using ethanol as a fuel is more friendly for the environmental than diesel engine than diesel that produce many various of air pollutant. This is because, ethanol has higher octane number that can reduce the content of harmful exhaust substances (Park *et al.*, 2011). Others advantages have been discussed by Yoo et al., (2011) are as follows:

I. Moreover, bio-ethanol blended with gasoline has an advantage in economy where estimate about 1 to 3% by increasing ethanol content compared to unleaded gasoline.
II. Ethanol-blended fuel improves the spark ignition performance of automatic engines.
III. Have superior anti-knock characteristic of over hydrocarbon fuels with a similar octane number .

However there are many obstacles of using ethanol as a fuel as listed:

i. Ethanol has limited solubility in diesel fuel. Phase separation and water tolerance in ethanol–diesel blend fuel are crucial problem.
ii. Ethanol fuel has an extremely low cetane number, whereas the diesel engine is prefer to high cetane number fuels which makes auto-ignition easily and gives small ignition delay.
iii. The dynamic viscosity of ethanol is much lower than that of the diesel fuel, so that the lubricity is a potential concern of ethanol–diesel blend fuel.

2.1.3 Acetone

Acetone is colourless, flammable liquid and miscible with water and an important solvent in industries. This organic compound has molecular weight 58.08 g/mol and molecular formula C_3H_6O. Acetone is widely used in industries to produce plastics and synthetic fibre. Acetone is the simplest member of ketone family and one of the abundant organic compound with free troposphere with concentration up to 0.5 ppb in clean air at northern mid-latitudes. Acetone has an important influence on the oxidising of the atmosphere and indirectly on cloud nucleation and the production of low level ozone (Harrison *et al.*, 2011).

Acetone is important as fuel tracer to the field of combustion (Pichon *et al.*, 2009). Recently, a ketal that produced from glycerol and acetone was used as a suspension agent in pharmaceutical preparations while in degradation of polymer and dehydration of carbohydrate, the acetone was used as sipercrritical fluid (Royon and Locatelli., 2011).

2.2 *Clostridium beijerinckii*

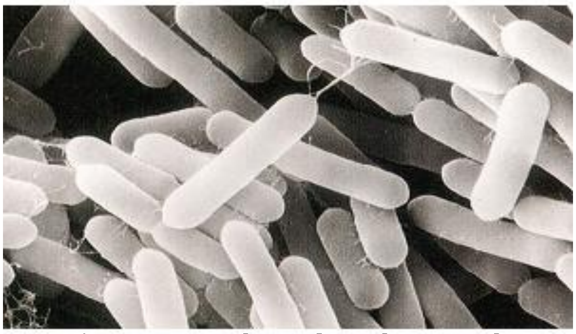


Figure 2.1: *Clostridium beijerinckii*

Clostridium beijerinckii is valuable bacterium. It from gram positive straight rod- shaped bacillus as can see at the picture above. This bacteria is typically anaerobes which can thrive without presence of oxygen, heteromenfertive (fermentation that are produces major product that are different) and spore-forming. Spore are oval and subterminal. Clostridia bacteria can be use to produced butanol. ABE clostridial strains are generally classified into 4 distinct group based on their biochemical and genetic characteristic. The best known group are *C. acetobutylicum* and *C. beijerinkii* and one of the most documented strain in ABE fermentation research studies (Karakashev *et al*, 2007). Mostly family of clostridia are highly pathogenic and can cause diseases. However, *C. beijerinckii* were harmless to people, animals and plants. Thus, is used in a wide range of chemical. *C. acetobutylicum* and *C. bijerinkii* are the one most documented (Qureshi *et al.*, 2010).

ABE fermentation by *Clostridium beijerinckii* can be divided into 2 phases which are acid production phase and solvent production phase. During acid production phase, the cell grows rapidly and form carboxylic acid which are acetate and butyrate that cause lowers in pH. These acid acts as inducer for the biosynthesis of the solventogenic enzymes during second phase. The acid formed during first phase re-enter cell and act as co-substrates for the neutral solvent production. At this point, acid production ceases as well as cell growth and pH in medium increased due to acid uptake. *C. beijerinckii* was able to metabolize a great variety of carbon resources. documented in ABE fermentation research studies (Karakashev *et al*, 2007). It is important to convert carbon residing in biomass to a liquid hydrocarbon that can be used in an internal combustion engine (Pfromm *et al.*, 2010).

At second phase, carbon and electrons are directed to the formation ABE solvent. At the end of the fermentation, solvent concentration may reach inhibitory level and halt the metabolism (Badr and Hamdy, 1992). The transition from acidogenic to solventogenic was accompanied by a decrease in culture fluorescence which was interpreted as a low intracellular NADH levels (Badr *et al., 2001*).

2.4 Palm Oil Mill Effluent (POME)

POME is generated from palm oil processing plant. POME, when fresh is a thick brownish in color colloidal slurry of water, oil and fine cellulosic fruit residues. POME is charged at a temperature of between 80°C and 90°C and it is slightly acidic with a pH between 4 to 5.Pome contain very high biological oxygen demand (BOD) and chemical oxygen demand (COD) which can cause pollution if not properly managed (Takriff *et al.*, 2009). POME is highly polluting due to its organic nature and its discharge to a relatively small river can be devastating to its ecosystem. Several attempts have been made in the bioconversion of these palm oil wastes into fertiliser, fuel, water reclamation, and citric acid (Jamal *et al., 2011*).

In fact, POME contained high concentration of carbohydrate, protein, nitrogenous compounds, lipid and minerals that recommended of reuse of POME as fermentation media (Wu *et al.*,2009). Kalil *et al.*, 2003, studied the direct use of POME as fermentation media for ABE production by *C. acetobutylicum* and immobilized C. saccharoperbutylacetonicum in a batch culture system. It was found that *C. acetobutylicum* produced the highest ABE in 90% sedimented POME with initial pH produced highest total ABE which is 4g/L (Kalil *et al.*, 2003). Takriff *et* *al*., 2009 cultured *C. acetobutylicum* in POME using oscillatory flow bioreactor to enhanced the production of ABE.

CHAPTER 3

METHODOLOGY

3.1 Equipment

3.1.1 Anaerobic Jar

Figure 3.1: Anaerobic Jar

Anaerobic jar is an equipment that can produce anaerobic environment. Anaerobic sachet must be added into the jar to obtain the anaerobic environment. One sachet is required to 2.5L jar. The sachet produce approximately 20% carbon dioxide and a residual atmosphere of less than 0.1% oxygen in one hour or less. The atmospheric oxygen in the jar is rapidly absorbed with the simultaneous generation of carbon dioxide. Moreover, water is not required to activate the reaction and it also does not required a catalyst. When used as directed, the sachet able to reduce the oxygen level in jar below 1% within 30minute.

3.1.2 Anaerobic Chamber

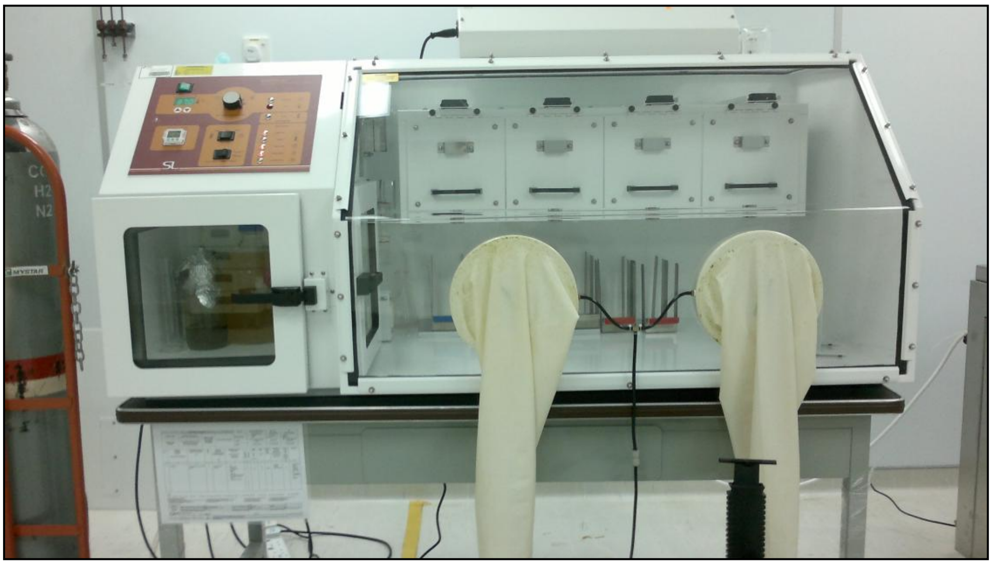


Figure 3.2 : Anaerobic Chamber.

Anaerobic chamber is another equipment used to attain anaerobe condition. The chamber parts include the flexible chamber with vacuum air lock and a system for continuous removal of oxygen in the chamber. It also consist a palladium catalyst containing 10% hydrogen that is functioning to remove trace of oxygen in the chamber by formation of water. High purity of nitrogen was used to purge the oxygen out of the chamber before used. The airlock is functioning to allows the samples without changes to the internal atmosphere. However, the small item can be brought in through the glove when enter the chamber. The chamber is also provided with temperature setting and temperature alarm. The alarm will sound to alert operator that the incubator has gone over set point. A proper use of the chamber can prevent a contamination air in the chamber.